Phosgene Effects on F-actin in Cells Grown from Pulmonary Tissues

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ABSTRACT

Confocal laser microscopy has been used to study the effects of phosgene on cells of the lung. Results suggest that the F-actin cytoskeleton is a molecular target and sensitive indicator of phosgene toxicity. Ovine pulmonary artery endothelial cells, exposed at 0.145 to 5.39 x LCT_{50} for sheep (3300 ppm · min) showed dose response decreases in F-actin content. Doses of 0.145 and 0.265 LCT_{50} caused a significant ($p < .01$) 25% and 42% decrease in average F-actin per cell. Dense peripheral bands (DPBs) became indistinct at $\geq$ 1.2 LCT_{50} and disappeared at $\geq$ 2.3 LCT_{50}. Organization of stress fibers was parallel to the cell's long axis and was not disrupted by < 1.21 LCT_{50}. In secretory cells from rat tracheal explants, studies indicate a threshold of resistance to phosgene at doses < 0.2 LCT_{50}. However, phosgene in excess of 0.2 LCT_{50} produced precipitous decreases in secretory cell F-actin. Mature, contiguous populations of untreated secretory cells contained well defined DPBs and tightly connected cell-to-cell boundaries. Exposures to 1.0 and 1.5 LCT_{50} did not disrupt boundaries between secretory cells but did cause separation of boundaries between secretory and other cell types. We conclude that concentration and organization are separate aspects of phosgene's effects on F-actin and that the lesions produced are cell-type specific.

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INTRODUCTION

Lung injuries from inhalation of phosgene and other respiratory agents became a threat to military personnel in World War I when U.S. Forces exposed to warfare gases numbered 70,552 injured and 1,221 killed (Gilchrist and Matz, 1933). Seventy-five years later, phosgene-induced lung injuries are once again a focus of medical attention and for reasons that transcend strict military usage. According to NIOSH (1976), more than 10,000 workers have been employed annually to manufacture 1 million tons of phosgene in essential support of our nations chemical industries. Recent reports indicate that 80,000 pounds are released each year into the ambient air (National Toxic Release Inventory, 1989). Clinical reports on occupational health suggest that respiratory injuries from work with paint removers and other chlorinated solvents may be due to phosgene inhalation and occur more frequently than commonly expected (Sjögren et al., 1991; Snyder et al., 1992). In accord with those figures and the potential threat of exposure, there is concerned interest regarding the threshold and mechanisms of phosgene induced toxicity. We know from the literature (Burleson and Keyes, 1989; Diller, 1985) that pulmonary edema and suppression of the lung's immune response system are primary causes of debilitation and death due to phosgene inhalation. Our studies suggest that the lesions affecting permeability and immunosuppression may be a function of phosgene's effects on cellular F-actin. The following study uses confocal laser microscopy to investigate these cytoskeletal effects.

MATERIALS AND METHODS

Lung Cells

Sheep pulmonary artery endothelial cells (PAEC) from low passage (#6-10), proliferating stock cultures were grown on glass coverslips which formed the bottoms of microwells drilled into 35 mm Petri plates. Populations seeded at 5000 cells/cm^2 were maintained by daily medium renewal for 3 days prior to phosgene gas exposure. Airway epithelial cells, grown from tracheal explants of male, Sprague-Dawley rats (*Rattus norvegicus*, CRL:CDBR VAF/Plus) were subject to medium renewal three times per week (M-W-F) and were in primary culture for a minimum of 3 weeks prior to phosgene exposure.

Culture Media

Nutrient media for sheep PAEC cultures consisted of HAM's F-12 with 15% FBS, endothelial cell growth factor (ECGF, 50 μg/ml), L-glutamine (2 mM), gentamicin sulfate (50 μg/ml), and 5% Omni™ (Advanced Biotechnologies Inc. Columbia, MD). Airway secretory cells were grown in HAM's medium supplemented with transferrin (10 μg/ml), insulin (5 μg/ml), epidermal growth factor (25 ng/ml) and 2% Omni™. Except for the Omni™ (serum supplement), all media and additives were products of the Sigma Chemical Co., St. Louis, MO.

Phosgene Exposures

Prior to gas exposure, populations were washed 3 times with 1.5 ml of HAM's F-12 medium without serum. Each culture was then covered with 0.5 ml of fresh HAM's F-12 to keep the cells wet during exposure. Replicate cultures were then placed in an environmentally controlled, stainless steel chamber and were exposed to phosgene for 20 minutes at 37 °C in a humidified atmosphere of 5% CO_2 and air. For the sake of comparison, doses (concentration x time) were based on LCT_{50} values for sheep, i.e., 3300 ppm·min (Keeler et al., 1990), and ranged from .145 to 5.39 x LCT_{50}. Doses were verified by gas chromatography on samples drawn from the chamber at the beginning and end of each exposure. Sham-treated controls were exposed to 5% CO_2 and air. Osmolarity of the supernatant fluid was checked before and after exposure using a model 5100 C vapor pressure osmometer (Wescor, Logan, UT). Viability was determined by nigrosin dye exclusion.

F-actin Staining

Control and phosgene-exposed populations were washed with PBS and fixed with 4.0% paraformaldehyde prior to F-actin staining. Fixed populations were washed with PBS, exposed to 70% acetone at -20 °C for 4 minutes, then permeabilized for 1.5 min. with 0.3% Triton X-100 and washed again in PBS. Cells were incubated for 90 min. at room temperature in FITC-phalloidin (3.99×10^{-7} M in PBS, Sigma Chemical CO. St. Louis, MO) and washed again in PBS. FITC-phalloidin is a fluorescent probe specific for F-actin.

Confocal Microscopy

Image analyses were performed on attached, freshly stained cell cultures using an ACAS-570 confocal laser cytometer (Meridian Instruments, Okemos, MI). Cells were scanned with a 488 line from a 5 watt argon laser. Excitation and emission spectra were separated with a blue, dichroic, beam splitting mirror that reflected wavelengths below 500 nm and transmitted wavelengths above 515 nm. Average fluorescence per cell (n = 42) was used to determine dose-response effects of phosgene on F-actin concentration. Line-scan analyses were used to determine how phosgene's effects on F-actin content affected its cytoskeletal organization.

Analyses of Data

For each of the populations studied (untreated controls, sham-treated controls and phosgene-exposed populations), 42 cell scans were processed from 2-3 replicate populations. Fluorescent emissions from replicate control and exposed populations were subject to one-way analyses of variance. Dose-response comparisons were made by Newman-Keuls multiple comparison tests. Population differences with $p < .01$ were considered significant.

RESULTS

Control Sheep PAEC

Microfilaments became well organized in sheep pulmonary artery endothelial cells. After 3 days in culture, images reproduced by 3-D reconstruction revealed an interwoven network of filamentous F-actin that generally conformed to the shape of these attached cells. Using non-invasive laser microscopy and confocal methods PAEC were sectioned as 0.5 μm slices throughout their z-axis. Dense peripheral bands (DPBs) and stress fibers were characteristic features of F-actin organization in these slices. Line-scan analyses show, as illustrated in Fig. 1, that peak emissions from DPBs were 3 to 4 times greater than emissions recorded from stress fibers and microfilaments in the cell cytoplasm. Line-scans also measured the distance or size of analyzed cells and DPBs, which in Fig. 1 were 21 and 2.0 - 2.5 μm respectively. Despite variations in cell shape, the image in Fig. 1 shows organization that was characteristic of F-actin in untreated and sham-treated control populations.

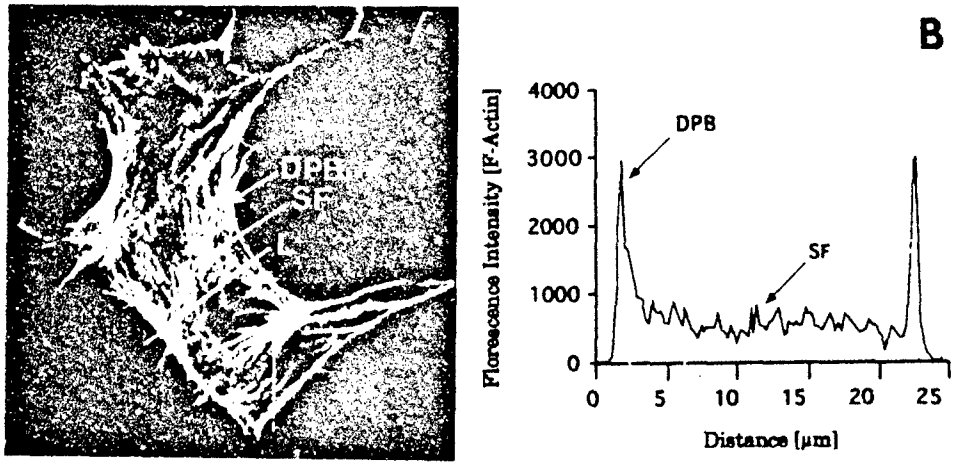

Fig. 1 Image of F-actin in sheep PAEC (A) shows organization of dense peripheral bands (DPBs) and stress fibers (SF). Line-scan analysis (B) shows fluorescent intensity and distribution of F-actin along the line of analysis (L).

Phosgene Effects on F-actin Organization

Line-scan analyses (Fig. 2) were used to show the following dose-response effects. At doses equivalent to 0.145 LCT_{50} for sheep (480 ppm · min), DPBs and stress fibers did not appear to be substantially altered immediately after exposure. They were intact and topographically in place but line-scan analyses detected a decrease in fluorescent emissions. Exposures of

1.21 LCT_{50} (3993 ppm min) did cause an immediate, post-exposure decrease in emissions from both DPBs and stress fibers. This dose did not, however, disrupt the position or orientation of DPBs and stress fibers within PAEC. With doses of 2.27 LCT_{50} (7491 ppm · min), microfilament organization was disrupted and line-scan analyses showed that the peak intensity of DPBs had diminished to the intensity of stress fibers. At 5.39 LCT_{50} (17,787 ppm · min), F-actin infrastructure was immediately and completely destroyed.

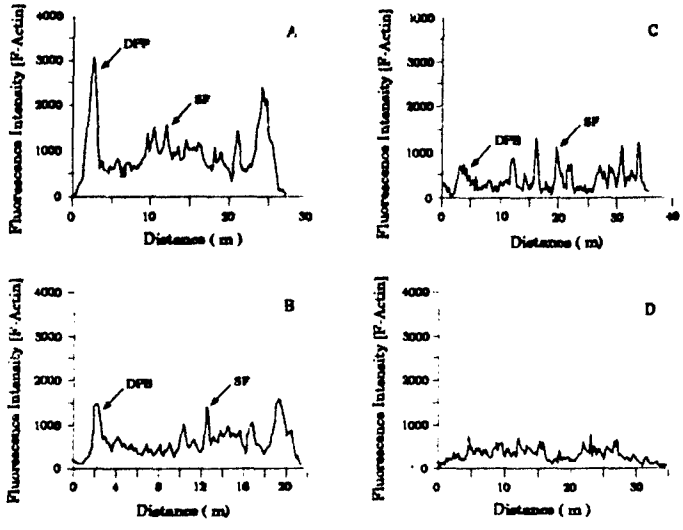

Fig. 2 Line-scan analyses through the midline of sheep PAECs exposed to phosgene at doses of (A) .145 LCT_{50}; (B) 1.21 LCT_{50}; (C) 2.27 LCT_{50} and (D) 5.39 LCT_{50}. Cells were fixed for analysis immediately after 20 minute exposures. Graphs show fluorescent intensity along each line of analysis and comparisons show the relative dose-dependent decrease of F-actin in DPBs and stress fibers (SF).

Phosgene Effects on PAEC F-actin Concentration

Phosgene produced immediate and corresponding effects on average F-actin content (Fig. 3). Sham-treated controls had 6% less F-actin but were not significantly different from untreated controls. Doses as low as 23 ppm x 20 min (0.15 LCT_{50}), however, produced an immediate and significant ($P < .01$) decrease in F-actin that was 25% less than sham-treated controls. With 44 ppm x 20 min (0.27 LCT_{50}), there was a 42% decrease in F-actin. At doses from 0.27 LCT_{50} to 2.27 LCT_{50}, we observed a threshold where the immediate post exposure decrement in F-actin remained at 39 to 45%. At 5.39 LCT_{50}, the threshold was exceeded and the dose caused a 79% decrease in F-actin. ANOVA shows significance (** = $p < .01$) for all doses compared to sham-treated controls. Newman-Keuls multiple comparison tests indicate a significant F-actin decrease (# = $p < .01$) at the beginning and end of the dose-response threshold (0.27 to 2.27 LCT_{50}).

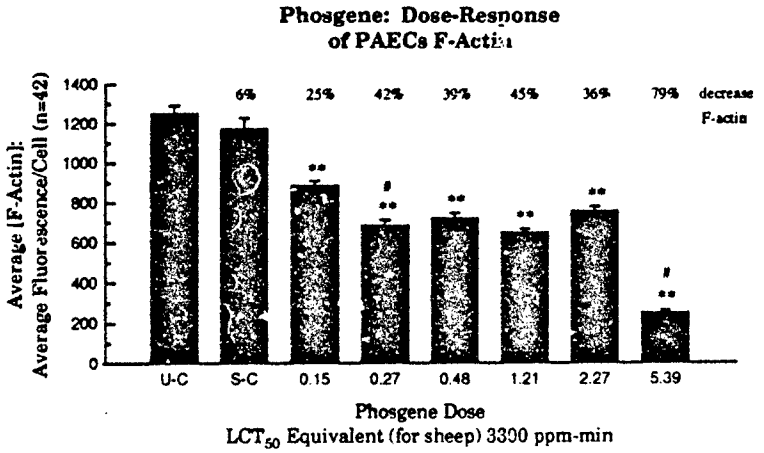

Fig. 3 Dose response bargraph showing effects of phosgene on average F-actin content in sheep PAEC. U-C = untreated control populations; S-C = sham-treated control populations. Doses are expressed as their LCT_{50} equivalent for sheep. ANOVA shows a significant F-actin decrease (** = $p < .01$) for all doses compared to sham-treated controls. Multiple comparisons show significance (# = $p < .01$) where results were compared to the previous dose.

Osmolarity was practically constant and remained between 304 and 312 mOsm during each of the 20-minute exposure periods.

Viability, even at the highest dose (5.39 LCT_{50}), was unaffected by phosgene. Nigrosin dye exclusion was > 97% for each of the doses tested and for each 1000 cells counted.

Effects of Phosgene on Rat Tracheal Secretory Cell F-actin.

In dose response studies with rat tracheal secretory cells, phosgene also produced an immediate and substantial decrease in average F-actin per cell (Fig. 4). At doses of 18 ppm x 20 min. (0.108 LCT_{50}), the decrease in average F-actin was only 8% compared to cells from untreated control populations. With 46 ppm x 20 min. (0.278 LCT_{50}), however, there was an immediate and significant ($p < .01$) decrease of 25% in average F-actin. At 131 ppm x 20 min. (0.794 LCT_{50}), the immediate post exposure effect was a 53% decrease in F-actin. In the dose range from 0.794 LCT_{50} to 3.2 LCT_{50} (528 ppm x 20 min), there was a threshold in which the immediate post exposure F-actin decrement held between 53% and 57%. Rat tracheal secretory cells were not subjected to higher doses. In Fig. 4, ANOVA show significance for all results (* = $p < .05$; ** = $p < .01$) compared to untreated controls. Multiple comparisons showed that when average results were compared to the previous dose, significant decreases in F-actin (# = $p < .01$) occurred at the lowest doses and prior to the threshold.

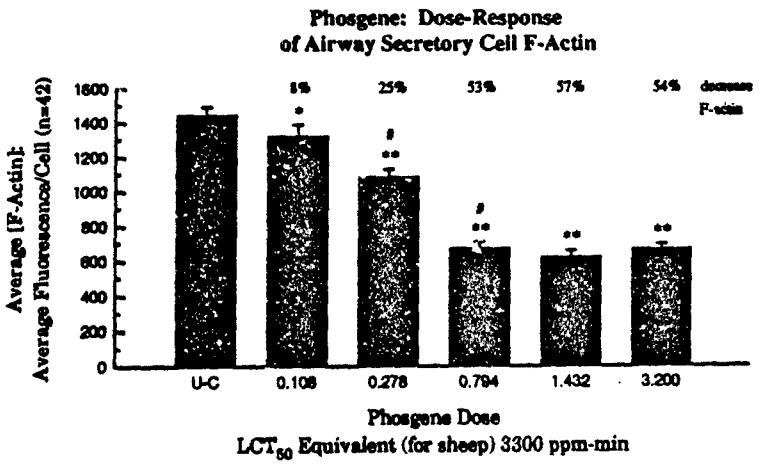

Fig. 4 Dose response bargraph showing effects of phosgene on F-actin of rat tracheal, secretory cells. U-C = untreated control population; Doses were based on LCT_{50} equivalent doses for sheep. * = $p < .05$, and ** = $p < .01$, used when F-actin content was compared to U-C; # = $p < .01$, used when F-actin data were compared with results obtained at previous dose.

F-actin Organization in Rat Tracheal Secretory Cells

At low population densities, the F-actin of proliferating, rat tracheal secretory cells lacked organization and was distributed in a random erratic pattern throughout the cell cytoplasm. Dense peripheral bands were not apparent, and it was difficult to distinguish between individual cells based on their F-actin cytoskeleton. In mature populations, F-actin formed DPBs that outlined the membrane margins of each cell in bold, distinctive patterns (Fig. 5).

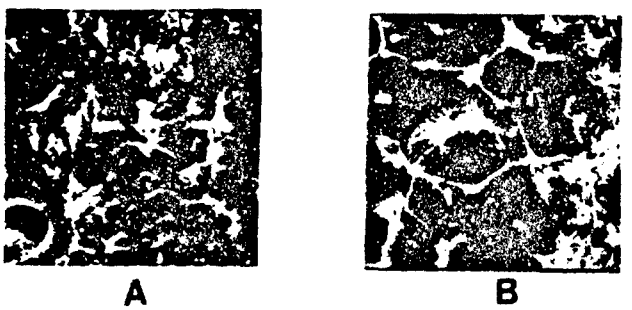

Fig. 5 F-actin in rat tracheal secretory cells. Proliferating, low density population (A), lacks F-actin organization. Mature, differentiated population (B), contains highly organized F-actin with bold, DPBs and tightly joined membrane margins.

Dense peripheral bands and the associated membrane junctions of these mature cells showed considerable resistance to phosgene exposures. Although the junctions between adjacent secretory cells were not immediately disrupted by phosgene, junctions between secretory cells and other cell types were less resistant and did tear apart during exposures.

DISCUSSION

Using confocal laser microscopy, we have demonstrated that phosgene has an immediate, dose-dependent effect on the concentration and distribution of cytoskeletal F-actin in cells grown from pulmonary tissues. Results from the above and related studies indicate that F-actin is a sensitive indicator of phosgene toxicity and that its response to phosgene appears to be cell-type specific. At a dose of 24 ppm x 20 min (0.15 LCT_{50}) there was a 25% decrease in the average F-actin of sheep PAEC. This dose is equivalent to data published on LCT_{50} values for rat and humans, i.e., 400-500 ppm · min (Diller and Zante, 1982). It is well below LCT_{50} values for sheep, i.e., 3300 ppm · min (Keeler et al., 1990) and is in excess of doses known to suppress the lung's immune response system, i.e., < 240 ppm · min, (Ehrlich and Burleson, 1991; Burleson and Keyes, 1989; Selgrade et al., 1989). With sheep PAEC the dose-response curve (Fig. 3) indicated a biphasic aspect to phosgene's effects on F-actin. At doses of 0.145 and 0.265 LCT_{50}, PAEC F-actin decreased sharply by 25% and 42% respectively. At higher doses of up to 2.27 LCT_{50} (7491 ppm · min), there was no further decrease in PAEC F-actin. The dose-response pattern of phosgene's effects on F-actin in rat tracheal secretory cells (Fig. 4) showed a similar biphasic effect. However, the doses required to produce comparable decreases in rat trachea, secretory cell F-actin, i.e., 25% and 53%, were roughly two-to-three times as high as those determined from the sheep PAEC, i.e., (0.278 and 0.794 LCT_{50}) vs (0.145 and 0.265 LCT_{50}), respectively. This result suggests that F-actin is a sensitive target molecule of phosgene in both species; however, cells of different species or different cells from the air-blood barrier may be slightly more or less resistant to phosgene. Therefore, phosgene's effects on cells of the lung and their corresponding function may be dose-dependent and cell-type specific. Evidence which may link phosgene's effects on the F-actin cytoskeleton to decreased barrier function and to changes in the lungs immune response system is also presented by Werrlein, Madren-Whalley and Kirby at this bioscience review.

CONCLUSION

The F-actin cytoskeleton is a molecular target and sensitive indicator of phosgene toxicity in cells grown from sheep and rat pulmonary tissues.

Concentration and organization are related but separate aspects of phosgene's effects on F-actin.

Phosgene's effects on F-actin are cell type specific.

REFERENCES

Burleson, G.R. and Keyes, L.L. (1989). Natural killer activity in Fischer-344 rat lungs as a method to assess pulmonary immunocompetence: Immunosuppression by phosgene inhalation. Immunopharm. Immunotox. 11(2 & 3): 421-443.

Diller, W.F. (1985). Pathogenesis of phosgene poisoning. Tox. Indust. Health 1(2): 7-15.

Diller, W.F. and Zante, R. (1982). Dosis-wirkungs-beziehungen bei phosgene-einwirkung auf mensch und tier. zbl. Arbeitsmed. 32(10): 360-368.

Ehrlich, J.P. and Burleson, G.R. (1991). Enhanced and prolonged pulmonary influenza virus infection following phosgene inhalation. J. Tox. Environ. Health 34: 259-273.

Gilchrist, H.L. and Matz, P.B. (1933). The residual effects of warfare gases: The use of chlorine gas, with report of cases. Med. Bull. VA 9: 229-270.

Keeler, J.R., Hurt, H.H., Nold, J.B., and Lennox, W.J. (1990). Estimation of the LCT_{50} of phosgene in sheep. Drug and Chem. Tox. 13(2 & 3): 229-239.

National Institute for Occupational Safety and Health (1976). Occupational exposure to phosgene. Publication No. 76-137 (NIOSH), US Dept. of Health, Education, and Welfare, US Government Printing Office, Washington, DC, pp 1-129.

National Toxic Release Inventory (1989). US Congress Subcommittee on Health and the Environment. Chairman: The Honorable Henry A. Waxman

Selgrade, M.K., Starnes, D.M., Illing, J.W., Daniels, M.J., and Graham, J.A. (1989). Effects of phosgene exposure on bacterial, viral, and neoplastic lung disease susceptibility in mice. Inhal. Tox. 1: 243-259.

Sjögren, B., Plato, N., Alexandersson, R., Eklund, A., and Falkenberg, C. (1991). Pulmonary reactions caused by welding-induced decomposed trichlorethylene. Chest 99: 237-238.

Snyder, R.W., Mishel, H.S., and Christensen, G.C. (1992). Pulmonary toxicity following exposure to methylene chloride and its combustion product, phosgene. Chest 101: 860-861.